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Expression and subcellular localization of mouse 20S proteasome activator complex PA28

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Abstract We have cloned the mouse PA28 proteasome activator cDNAs. Northern blot demonstrates high PA28 mRNA levels in liver, kidney and lung. mRNA levels are low in thymus, spleen and brain. In contrast, PA28 protein levels vary little between these tissues. Immunocytological analysis and cell fractionation experiments demonstrate that both subunits are almost equally distributed between the cytoplasm and the nucleus. Interestingly, PA28α spares nucleoli, while PA28β is strongly enhanced in the nucleolus. This indicates for the first time that the PA28α and PA28β subunits may serve nuclear functions which may be different from and independent of each other.

Key words: Proteasome; Activator; Ki-autoantigen

1. Introduction

The 20S proteasome is the major cellular extra-lysosomal protease of eukaryotic cells [1]. This 700 kDa proteolytic complex consists of 14 non-identical subunits ranging in molecular mass from 21 to 31 kDa [1]. The subunits can be classified as α and β type according to their homology to the α and β subunits of the ancestral 20S proteasome of the archaebacterium *Thermoplasma acidophilum* [2]. Three of the β subunits possess a free N-terminal threonine thought to be involved in the formation of the active sites which are oriented towards the lumen of the cylinder [3,4].

Being involved in such different processes such as cell division, antigen processing, transcription factor activation and protein turnover [5–7] proteasome activity can be modulated through the interferon-γ (IFN-γ) induced exchange of specific β subunits or the interaction with regulatory proteins.

In the presence of ATP the 20S proteasome binds a 19S regulator complex (PA700) composed of about 15–20 different non-proteolytically active subunits to form the 26S proteasome responsible for the ATP–ubiquitin-dependent pathway of protein degradation [7]. PA700 which binds to the two α endplates of the 20S proteasome [8] appears to be responsible for the recognition of multi-ubiquitinated protein substrates via subunit S5a [9]. PA700 contains also subunits with ATPase activities and subunits which cleave multi-ubiquitin chains during proteolysis [10].

In contrast to PA700, PA28 or 11S regulator associates with the 20S proteasome in an ATP-independent manner. PA28 stimulates the hydrolysis of small fluorogenic peptide substrates [11,12] and influences the processing of larger synthetic polypeptide substrates in vitro [13]. The in vivo association of PA28 and 20S proteasomes was demonstrated by Yang et al. [14]. The hexameric PA28 is formed by two non-identical subunits named PA28α and PA28β. In SDS-PAGE the subunits reveal an apparent molecular mass of 31 kDa (α) and 29 kDa (β) respectively [15–17]. They form ring-shaped hexameric complexes with an approximate molecular mass of 180 kDa [16,18] which bind to the α-endplates of the 20S proteasome [19,20]. A stoichiometry of three α and three β PA28 subunits per hexameric complex has recently been proposed [18,21,22]. As opposed to the 26S proteasome little is known about in vivo substrates of the PA28-20S proteasome complex. The fact that PA28 α and β as well as the proteasome subunits LMP2, LMP7 and MECL-1 are up-regulated by INF-γ [16,23,6] suggested that PA28 participates in the cellular immune response. In support of this we recently were able to show that over-expression of PA28α enhances MHC-class I antigen presentation in mouse B8 fibroblast cells [24].

In the present communication we have analysed the mouse PA28. We cloned the PA28α and PA28β cDNAs and based on the deduced amino acid sequences we have raised subunit-specific antibodies. The subcellular distribution of PA28α, PA28β, and Ki autoantigen, the latter shares considerable sequence homology with the two activator subunits in mouse fibroblast B8 cells as well as the expression of the two PA28 subunits in different mouse tissues at the RNA and protein level was investigated. Our data show that mRNA levels of both PA28 subunits vary depending on the tissue analyzed. This variation is not reflected at protein level. PA28 subunits are present in the cytoplasm as well as in the nucleus while the Ki autoantigen, which exhibits extensive sequence identity with the PA28 subunits, is found exclusively in the nucleus. Interestingly, the nuclear staining patterns for PA28α and PA28β are not identical and PA28β, in contrast to PA28α, seems to accumulate in the nucleolus. These data may indicate a so far unknown nucleus-specific function for PA28β which is independent of PA28α.

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Abbreviations: PCR, polymerase chain reaction; MHC, major histocompatibilty complex; IFN-γ, interferon-γ; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid

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2. Materials and methods

2.1. Cloning of the PA28α and PA28β subunits of mouse PA28 regulator

The mouse PA28α cDNA was isolated and cloned as previously described [24]. The mouse cDNA encoding the PA28β subunits was isolated from a mouse spleen λ-ZAP II phage library (C57 Black/6, Stratagene, Heidelberg). About 2×10^6 recombinants were screened by plaque hybridization. A [α-^{32}P]dATP-labelled PA28β rat cDNA [16] probe was used for hybridization. Hybridization conditions were as follows: hybridisation buffer was 6×NaCl/Cit. (1×NaCl/Cit. is 0.15 M NaCl, 0.003 M sodium citrate, pH 7.0), 5×Denhardt, 0.1% SDS, 10 mM EDTA, 100 μg/ml salmon DNA, 60°C overnight. The nitrocellulose filters were washed 2 times with 2×NaCl/Cit., 0.1% SDS at room temperature for 20 min, and 2 times with 0.2×NaCl/Cit., 0.1% SDS at 60°C for 20 min. The PA28β cDNA was excised by helper phage following the manufacturers' protocol.

2.2. Antibody production

Polyclonal mPA28α, mPA28β and human Ki antigen peptide antibodies were raised by immunizing rabbits with KLH-coupled synthetic peptides. For the mPA28α subunit residues 5-19 (RVHPEAQAKVDVFRE) and for the mPA28β subunit residues 16-31 (KQVDVFRQNLFQEADDF) were used. For the human Ki-antigen [16] we used amino acid residues 70-86 (ILLTNSHDGLDGGPTYK). The polyclonal mPA28βrec antibody was generated from a PCR fragment of 276 bp encoding amino acids 1–92. The PCR fragment was cloned into a pQE6×His vector (Qiagen, Hilden, Germany). Cells were grown in LB (10 g bacto-tryptone, 5 g bacto yeast extract, 10 g NaCl) to an OD_{600} of 0.8 and expression was stimulated by addition of isopropyl-β-D-thiogalactopyranoside (4 h, 37°C) to a final concentration of 1 mM. The expressed recombinant protein was purified on a Ni-column purification system following the denaturing purification protocol described by Qiagen. Fractions (2 ml) were collected and analysed by SDS-PAGE. Purified recombinant protein was used for antibody production in rabbits. For immunofluorescence experiments all antibodies used were purified by affinity chromatography on antigen coupled to epoxy-activated Sepharose 6B (Sigma) columns.

2.3. Preparation of protein extract from mouse tissues and protein extracts from nuclei and cytoplasm of B8 cells

Different mouse tissues were homogenized under liquid nitrogen. Homogenized tissue (100 mg) was lysed in 50 mM Tris-HCL (pH 7.5), 5 mM $MgCl_2$, 0.1% Triton-×100, 1 mM EDTA; 6 mM aprotinin, 7 mM pepstatin, 1 mM PMSF; 10 mM leupeptin and homogenized by using a teflon potter. After centrifugation at 20 000×*g* at 4°C for 15 min. The supernatant was mixed with 4×SDS-sample buffer. Supernatant protein (60 μg) was separated by conventional 15% SDS-PAGE. Protein concentrations were determined as described by Bradford [25]. Preparation of cytosolic and nuclear protein extracts from B8 cells was performed as described by [26].

2.4. Immunoblotting and electrophoretic methods

Discontinuous tricine–SDS-PAGE system was carried out as described [27]. Following SDS-PAGE gels were transferred onto nitrocellulose. Antigen–antibody complexes were detected by Western blotting. For immuno-detection the BM Chemiluminescence kit from Boehringer Mannheim was used.

2.5. RNA preparation and Northern blot

Total RNA was isolated from mouse tissues using the RNA easy Kit from Qiagen. Separation on formaldehyde containing agarose gels, transfer onto membranes and radioactive probing were carried out as described previously [28].

2.6. Cell culture

B8 fibroblast cells were grown in IMDM containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 400 μg/ml G418 (Gibco).

2.7. Immunofluorescence analysis of B8 cells

Cells were fixed in 3.7% paraformaldehyde for 10 min, washed in quenching buffer (50 mM NH_4Cl in PBS) and permeabilized with 0.1% NP-40 in washing buffer (PBS+0.01% saponine+0.25% gelatine) for 30 min at room temperature (RT). After incubation with the first antibody (mPA28αp 1:20; mPA28βp 1:2 or hKi peptide antibody 1:500) for 30 min at RT in a moist chamber, cells were washed 3 times for 10 min with washing buffer. The second fluorescein-conjugated antibody (goat anti-rabbit IgG, Jackson Immunoresearch Laboratories, West Grove) was diluted 1:400 in FCS. The cells were incubated for 30 min at RT. Subsequent washing steps were performed as described before. Cells were embedded and placed onto a glass slide. For competition immunofluorescence assays affinity purified mPA28αp, and mPA28βp antibodies were pre-incubated at 4°C overnight with purified human PA28 at a molar ratio of 3:1.

2.8. Double immunostaining

Double immunostaining was performed essentially as described [29]. Fixed and permeabilized cells were washed and blocked with 30% FCS for 15 min. Antibody incubations were performed for 30 min in a humid chamber followed by three washing steps 10 min each. After incubation with the first PA28 antibody (rabbit anti-mPA28αp) cells were incubated with fluorescein-conjugated Fab fragment goat anti-rabbit IgG (H+L) (Jackson Immunoresearch Laboratories, diluted 1:300 in FCS). Subsequently PA28β was detected by the rabbit anti-mPA28βp antibody which was stained by a Cy3-conjugated donkey anti-rabbit IgG (H+L) (Jackson Immunoresearch Laboratories, diluted 1:400).

Microscopy was done with a Leica DMRXE microscope equipped with a 63× plan apochromat objective. For photography Ilford XP2 400 ASA film was used.

3. Results

3.1. Cloning and sequencing of mouse PA28α and PA28β

To allow the generation of subunit-specific antibodies directed against the mouse proteasome activator PA28 α and β subunits and for the analysis of tissue-specific expression at both, the protein and mRNA level we isolated the corresponding cDNAs from a mouse λgt11 liver cDNA and a mouse λZAP-spleen cDNA library (see Section 2). The amino acid sequence deduced from the longest open reading frame of mPA28α cDNA (Fig. 1A) corresponds to a protein of 249 amino acids with a calculated molecular mass of 28 676 Da. Its sequence identity with the corresponding rat and human subunits is 95% and 91%, respectively (Fig. 1B).

The mPA28β cDNA contains an open reading frame of 717 nucleotides encoding a protein of 239 amino acids. The calculated molecular mass for the mouse β subunit is 27 075 Da (Fig. 1A). The mouse PA28β protein is 94% and 87% identical to the corresponding rat and human subunits, respectively (Fig. 1C). The mPA28α and mPA28β proteins are 50% identical to one another (Fig. 1A). Interestingly, an alignment of murine PA28α and PA28β shows a homogeneous distribution of homology throughout the protein except for a gap of 13 amino acids in PA28β. The region which is truncated in PA28β corresponds to the KEKE motif in PA28α which has been proposed to mediate protein–protein interaction of the 20S proteasome and the PA28 complex [30].

3.2. Analysis of mPA28α and mPA28β mRNA and protein levels in different mouse tissues

To determine the tissue specificity of mPA28α and mPA28β gene expression, we examined the levels of PA28α and PA28β mRNAs in different mouse tissues. Northern blot analysis shows that the mRNA levels of both subunits vary significantly between the various mouse tissues (Fig. 2A,B). The mRNA levels for both subunits were related to the amount of β-actin mRNA on the same Northern blots (Fig. 2C). A densitometric analysis of the Northern blots revealed that the mRNA levels of both subunits are relatively high in liver,

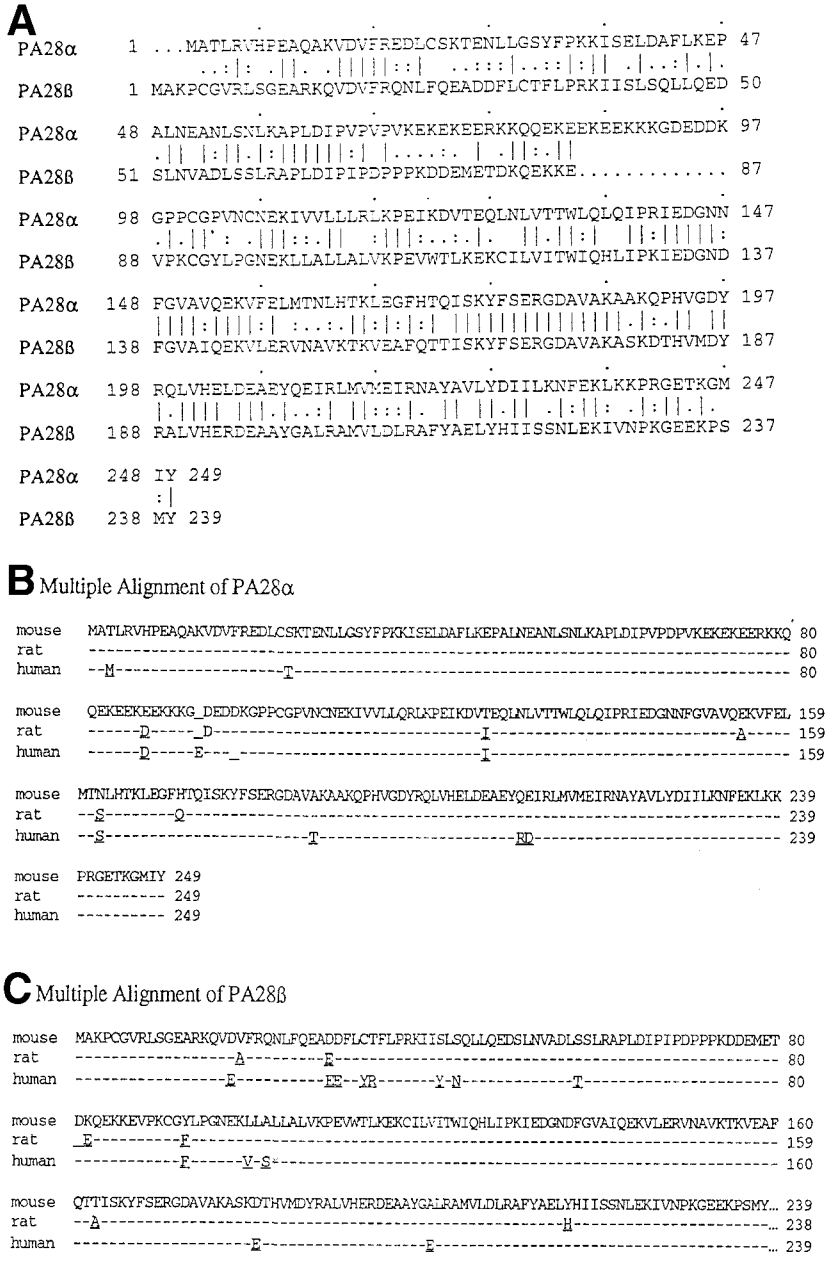

Fig. 1. Deduced amino acid sequences of the mouse PA28 α and β subunits and sequence comparison. The protein sequences of the mouse PA28α and β subunits were deduced from the longest open reading frames of the corresponding mouse cDNAs. A: Comparison of PA28α and PA28β protein sequences from mouse; comparison of the PA28α (B) and PA28β (C) amino acid sequences from mouse rat and man.

kidney and lung while thymus and spleen contain significantly less PA28α and PA28β mRNA. Interestingly, only negligible levels of PA28α and PA28β mRNA are found in brain (Fig. 2D).

Since mRNAs as well as the corresponding protein products can be controlled at post-transcriptional or post-translational level low amounts of mRNA do not necessarily reflect low levels of the corresponding proteins. Therefore, we separately determined PA28α and β protein quantities in various mouse tissues. To differentiate between the PA28α and PA28β subunits we raised subunit-specific polyclonal antibodies. A synthetic peptide covering amino acid residues 5-19 was used for the production of PA28α-specific antibodies (mPA28αp) and a PA28β-specific antibody (mPA28βrec) was obtained with a recombinant protein representing the N-terminal part of the protein (see Section 2). The subunit specificity of the antibodies was tested and documented by ECL immunoblotting (Fig. 3D). In agreement with the calculated molecular mass of 28.6 kDa for the α and 27 kDa for the β subunit and in contrast to recently reported data [21] our analysis of PA28α and PA28β protein migration in tricine–SDS-PAGE demonstrate that the upper of the two activator proteins is recognized by the antibody mPA28αp and that mPA28βrec identifies only the faster migrating protein which consequently represents the PA28β subunit.

Equal amounts of total protein from different mouse tissues were loaded in each lane, separated by SDS-PAGE and transferred onto nitrocellulose membranes. As shown in Fig. 3A,B,

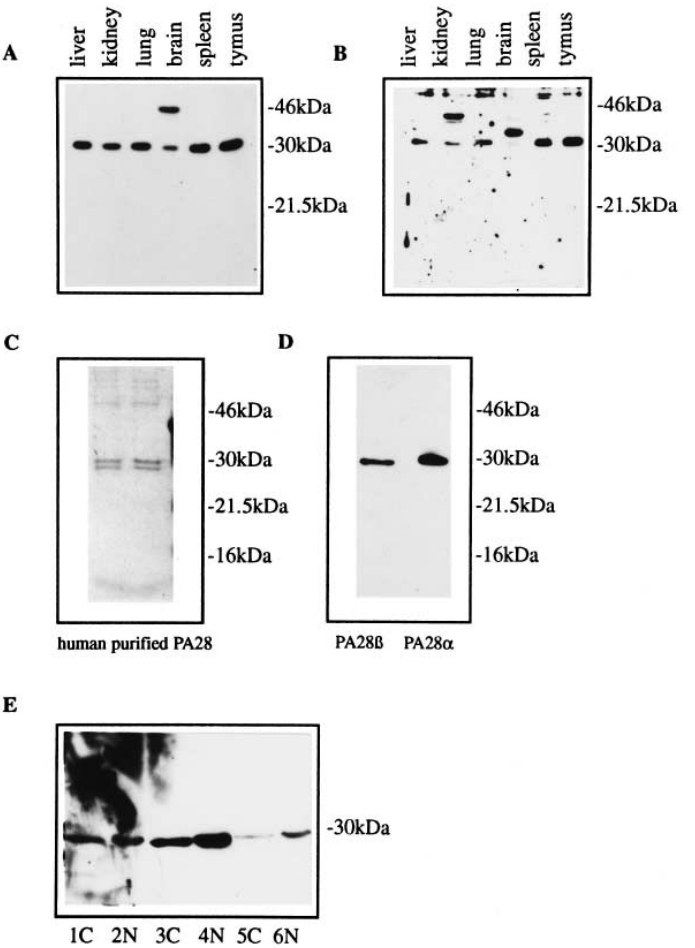

Fig. 2. Analysis of PA28α and β mRNA levels in different mouse tissues by Northern blot analysis. 15 μg of total RNA of different mouse tissues were loaded onto each lane. The mPA28α and mPA28β mRNA accumulation was detected using [α-^{32}P]dATP-labelled cDNA fragments of mPA28α (A) and mPA28β (B), respectively. A β-actin cDNA fragment was used as a control (C). Densitometric analysis of the Northern blots and adjustment of the values to the β-actin standard (D).

subunits PA28α as well as PA28β are expressed in all examined tissues. However, in contrast to the differences in mRNA accumulation in these tissues PA28α and PA28β protein levels do not significantly vary. Interestingly, when compared with the other tissues, thymus and spleen possess even increased amounts of PA28 protein in spite of a low PA28α and PA28β mRNA expression.

In conventional SDS-PAGE both, PA28α and PA28β, migrate at a position of 30 kDa in most murine tissues (Fig. 3A,B). A surprising exception was noted when Western blots were probed with the PA28β-specific antibody: in murine brain only a faint band was detected at 30 kDa when compared to other tissues and instead a prominent band appeared at 33 kDa. This finding was highly reproducible even when affinity purified mPA28βrec was used. Moreover, a brain-specific 33 kDa protein was also detected with a polyclonal PA28 antibody raised against purified human PA28 [17] (designated 'hPA28') but not with our PA28α-specific reagent. A fortuitous cross-reactivity with an unrelated brain-specific protein is therefore unlikely. Rather our results suggest that PA28β exists in a strongly modified form or that a tissue-specific larger isoform of PA28β is expressed in the murine brain. Covalent modification of PA28β with phosphate or charged sugars, however, are unlikely to account for the observed increase in molecular mass as we did not detect a charge difference in PA28β from mouse liver and brain in Western blots of two-dimensional IEF/SDS-PAGE (data not shown).

Further bands of about 45 kDa were detected by the mPA28αp antibody in brain and by the mPA28βrec antibody in kidney, but as these bands were not detected with the PA28 polyclonal antibody, we cannot be certain whether the detected proteins are related to PA28 subunits or whether they are cross-reacting organ specific proteins.

3.3. *PA28α and PA28β subunits are localized in the cytoplasm as well as in the nucleus*

To analyze the subcellular distribution of PA28α and PA28β subunits in mouse fibroblast B8 cells, we used affinity purified antibodies for indirect immunofluorescence microscopy (Fig. 4) and for Western analysis of cytoplasmic and nuclear fractions (Fig. 3E). The specificity of the applied antibody preparations was confirmed by competition experiments in immunofluorescence microscopy as a pre-incubation of the mPA28αp and mPA28βrec antibodies with purified PA28 complexes reduced the fluorescence signal almost to background levels (not shown). Immunostaining of mouse B8 cells demonstrates that both PA28 subunits are evenly distributed between the cytoplasm and the nucleus of the fibroblast cells.

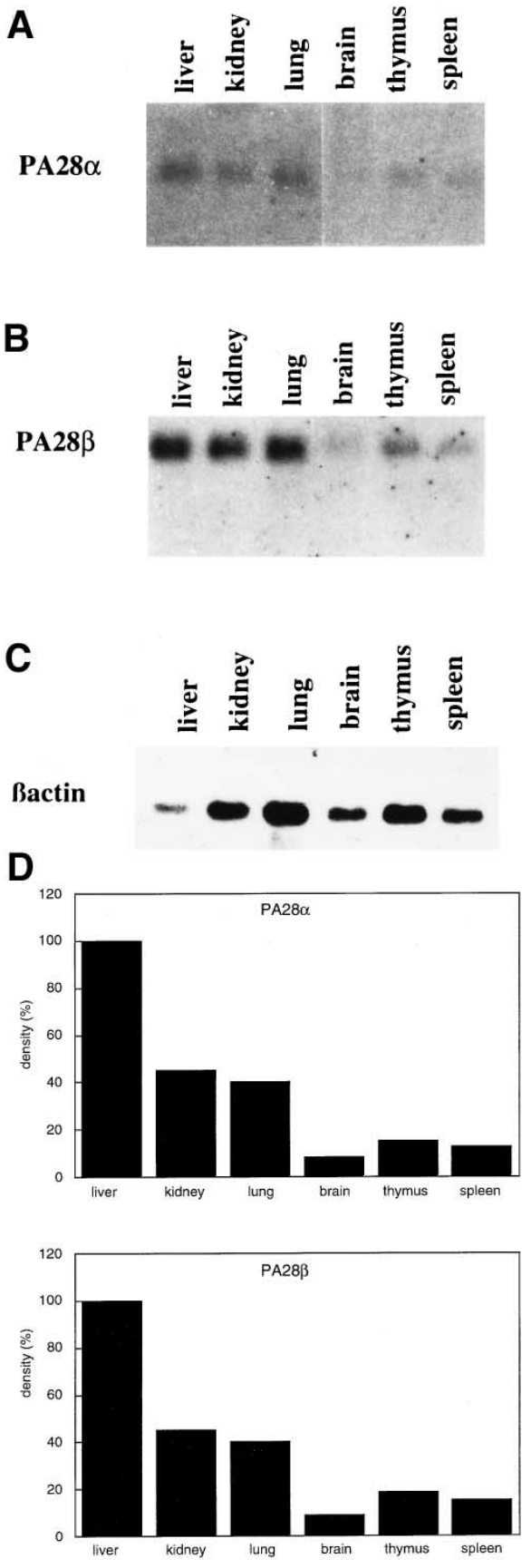

Fig. 3. Western blot analysis of PA28α and β protein subunit identity and protein levels in different mouse tissues. A: Immunoblot analysis of PA28α. B: Immunoblot analysis of PA28β. 60 μg of protein extract from different mouse tissues were loaded onto each lane. For immunoblotting the antibodies mPA28αp and mPA28βrec were used. C: Coomassie-stained blot of purified human PA28 (1.5 μg/lane) after separation on a discontinuous tricine–SDS-PAGE prepared according to Schägger and von Jagow [27]. D: Assignment of PA28α and PA28β subunits on purified and immunoblotted human PA28. The Western blots shown in (D) were probed with antibodies mPA28αp (right) and mPA28βrec (left). E: Identification of mouse PA28 subunits in nuclear and cytoplasmic extracts of mouse B8 fibroblast cells. 30 μg of protein from the crude cytoplasmic (C) and crude nuclear (N) fraction of B8 cells were separated on 15% SDS PAGE. PA28 subunits were identified by immunoblotting with affinity purified antibodies mPA28αp (1C, 2N), mPA28βrec (3C, 4N) and mPA28βp (5C, 6N).
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PA28α staining reveals a slight accumulation of the antigen in the nucleus where a speckled immunofluorescence image is observed. The nucleoli of the fibroblast cells are still visible as only weakly stained hollows (Fig. 4A). In contrast to the α subunit, PA28β immunostaining reveals particulate nuclear immunofluorescence signals (Fig. 4B). The distribution of PA28β fluorescence is consistent with an accumulation of PA28β in nucleoli. When mouse glia cells were analysed an identical fluorescence pattern was observed demonstrating that this result is not peculiar to mouse fibroblast cells (Stohwasser, personal communication).

In order to compare the intracellular distribution of the homologous Ki autoantigen to that of PA28α and PA28β we raised polyclonal antibodies in rabbits against a synthetic peptide corresponding to a region in the Ki sequence which lacks homology to PA28α and PA28β. Fig. 4C shows that Ki fluorescence is found exclusively in the nucleus leaving the presumed nucleoli as unstained hollows while the cytoplasm was completely devoid of Ki fluorescence. This result is in agreement with the existence of a nuclear targeting site in the Ki sequence which resembles that of the SV40 large T protein [31] and with the initial description of Ki as a nuclear antigen [32]. It also demonstrates that the affinity purified Ki antibody does not cross-react with PA28α or PA28β.

Taken together we conclude from these experiments that PA28 subunits are present in the cytosol as well as in the nucleus and that in contrast to existing biochemical evidence the PA28 subunits PA28α and PA28β do not always co-localize in the nuclear compartment. Furthermore, these data suggest that in addition to modulating the 20S proteasome activity the two PA28 subunits may possess mutually independent functions.

The observation, that PA28 antigens are predominant in nuclei was surprising. Nevertheless, the immuncytological data are compatible with immunoblotting experiments in which cytosolic and nuclear extracts of B8 cells were screened with PA28 antibodies (Fig. 3E). These experiments reveal that the PA28α subunit is present in the nuclear and cytosolic fractions at approximately equal amounts while the PA28β subunits is even slightly enriched in the nuclear fraction.

4. Discussion

In the present study we cloned the cDNAs encoding the two mouse proteasome activator PA28 subunits and analysed the PA28 mRNA as well as protein expression levels in different

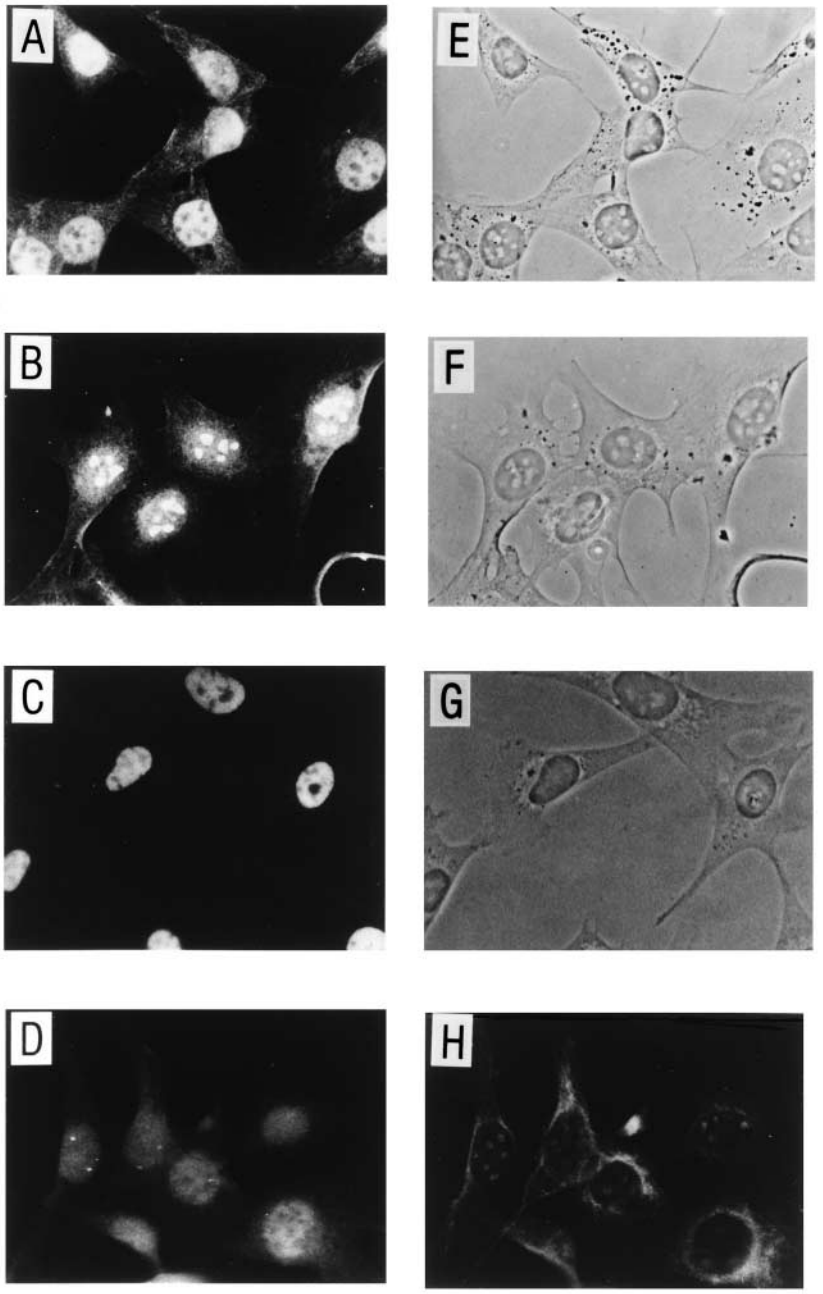

Fig. 4. Subcellular distribution of mPA28α and mPA28β protein subunits and the Ki autoantigen by fluorescence microscopy of mouse fibroblast B8 cells. For immunodetection of mPA28α and mPA28β paraformaldehyde-fixed B8 cell were used for indirect immunofluorescence. A: Immunodetection of the PA28α subunit with affinity purified mPA28α peptide antibody. B: Immunodetection of the PA28β subunit with affinity purified mPA28β peptide antibodies. C: Immunodetection of the Ki antigen with affinity purified antibodies. Pictures E, F, G represent phase contrast images of A, B, C. Lower panel shows double immunostaining of PA28α and β. D: PA28α; H: PA28β.

mouse tissues. In addition, we show for the first time the intracellular distribution of both the α and β subunits by immunofluorescence microscopy.

The primary structures of the mouse PA28α and PA28β subunits are 50% identical to each other whereas the mPA28 α subunit is 97% (93%) and the mPA28β subunit 93% (87%) identical to the corresponding rat and human activator proteins [16,33]. These high sequence identities support the suggestion [16] that the α and β type PA28 genes originate from a common ancestor.

Both subunits of the proteasome activator PA28 as well as the non-constitutive proteasome β-subunits LMP2, LMP7 and MECL-1 are up-regulated by INF-γ. Following induction, the three proteasome β subunits which modulate the proteolytic activities of the enzyme complex replace their constitutive homologues forming proteasome complexes which appear to be more appropriate for the processing of endogenous antigens presented by MHC class I molecules [6,34]. The mRNA levels of the non-inducible proteasome subunits were shown to be more or less constant in different tissues [1]. This differs to the IFN-γ-inducible subunits LMP2, LMP7 and MECL-1 whose constitutive steady-state mRNA levels were

shown to vary strongly between different mouse tissues [35,36]. In particular thymus and spleen, two tissues which are tightly connected with the immune response, exhibit high constitutive levels of LMP and MECL-1 mRNAs. In complete contrast, mRNA levels of mPA28α and mPA28β appear to be particularly low in thymus and spleen. Even lower amounts of PA28 mRNA are only found in brain. This striking difference in the constitutive levels between the two groups of mRNA may indicate that IFN-γ-inducible proteasome β-subunits and factors enhancing proteasome activity underlie, in the absence of the cytokine, different transcriptional regulatory mechanisms. Alternatively, there exists also the possibility that in different tissues the two groups of mRNAs possess different half-lives and that PA28 mRNAs are relatively short lived in these tissues.

In a recent publication there has been some confusion with regard to which of the two PA28 proteins represent the α and β subunits [21]. Our immunoblotting experiments provide clear evidence that in accordance with the deduced molecular mass and in agreement with data of Kühn and Dahlmann [17] the upper protein band represent the α and the faster migrating protein the β subunit of PA28.

Interestingly, our Western blot analysis of PA28 protein levels in the different tissues shows that in comparison to the low mRNA levels large amounts of the corresponding mPA28α and mPA28β proteins are detected in brain, spleen and thymus. Thus it appears that the low amounts of PA28 mRNAs are compensated either by increased protein stability or by translation efficiency. It is notable that despite the variations in PA28 mRNAs the relative PA28 protein levels are very similar in the various tissues. Only in thymus and spleen the amount PA28α and β protein is somewhat higher than in the other tissues analyzed here.

Our experiments also show that in brain the PA28β subunit exhibits a slightly lower electrophoretic mobility than that of the other tissues and that no protein corresponding to the electrophoretic mobility was found for this subunit in the other tissues could be detected. The PA28β subunits of reticulocytes as well as human erythrocytes were recently shown to be phosphorylated [37] but a brain-specific hyper-phosphorylation of PA28β appears to be unlikely as we did not observe a shift towards a negative charge in the larger PA28β protein from murine brain. Alternatively the difference in molecular mass of PA28β may reflect the existence of a tissue-specific isoform of the β subunit. The presence of distinct tissue-specific isoforms for proteins of the proteasome system was recently documented in Drosophila [38]. The latter possibilities are currently under investigation in our laboratory. Additional proteins with an apparent molecular mass of 45 and 55 kDa were detected before in all examined tissues [39]. In our blotting experiments we also detected proteins of approximately 45 kDa in extracts of kidney and brain. However, since these proteins were not detected with antibody hPA28 we regard these signals as unrelated to the PA28.

For a number of mammalian tissues, cultures of vertebrate cells and invertebrates 20S proteasome complexes were shown to be localized in the cytoplasm as well as in the nucleus [1]. In fact, dependent on the metabolic state of certain tissues or cell types the subcellular distribution of proteasome complexes appears to be differently regulated [40,41]. Strong nuclear localization of proteasomes was observed in rapidly dividing cells [40] and Peters and coworkers [42] showed that the nuclear immunofluorescence of 26S proteasome was more intense than in the cytoplasm. Only nucleoli and metaphase chromosome remained unstained. Our analysis of the subcellular distribution of PA28 in mouse fibroblast cells reveals that the activator subunits are distributed almost equally between the nucleus and the cytoplasm a result which is also supported by cell fractionation experiments. Similar results were obtained for mouse microglia (Stohwasser, personal communication) excluding the possibility that this represents a peculiarity of B8 cells. As judged from the immunofluorescence and immunoblotting experiments performed here, which reflect the steady-state situation in fibroblast cells, the amount of PA28 proteins present in the nucleus is considerably higher than the 10% reported by Ahn et al. [21] which immunoprecipitated PA28 from subfractions of pulse-labelled Hela cells.

Our results also reveal that the α and β subunits of PA28 do not completely co-localize in the nucleus and that they are present in different nuclear substructures. The β subunit is shown to strongly accumulate in the nucleoli whereas the α subunit does not accumulate in these sites and rather occupies the nucleoplasm. Although immunocytological data always should be interpreted with care these experiments indicate for the first time that the α and β subunits of PA28 may exists independent from each other and that they may serve different functions in the nucleus. Whether both subunits can interact with other proteins in vivo is at present unknown. For the α-subunit it has been shown that the recombinant subunit is able to activate proteasomal peptide hydrolyzing activity and to form complexes in vitro [30]. However, there is no evidence that such complexes are also formed in vivo [21]. Furthermore, in vitro the β-subunit on its own is not able to activate the hydrolyzing activity of the 20S proteasome complex [18]. Thus it appears that both proteins may require other partners to exert their in vivo function. Whether both proteins, besides forming PA28 complexes with each other, possess indeed additional protein partners in the nucleus or nucleolus must be a subject of further investigations.

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References

[1] Tanaka, K., Tamura, T., Yoshimura, T. and Ichihara, T. (1992) New Biol. 4, 173–187.
[2] Zwickl, P., Kleinz, J. and Baumeister, W. (1994) Struct. Biol. 1, 765–769.
[3] Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W. and Huber, R. (1995) Science 268, 533–539.
[4] Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R. and Baumeister, W. (1995) Science 268, 579–582.
[5] Hershko, A. (1996) Adv. Exp. Med. Biol. 389, 221–227.
[6] Groettrup, M., Soza, A., Kuckelkorn, U. and Kloetzel, P.M. (1996) Immunol. Today 17, 429–435.
[7] Coux, O., Tanaka, K. and Goldberg, A.L. (1996) Annu. Rev. Biochem. 65, 801–847.
[8] Peters, J.M., Harris, J.R. and Kleinschmidt, J.A. (1991) Eur. J. Cell. Biol. 56, 422–432.
[9] Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061.
[10] DeMartino, G.N. and Slaughter, C.A. (1993) Enzyme Prot. 47, 314–324.
[11] Chu-Ping, M., Willy, P.J., Slaughter, C.A. and DeMartino, G.N. (1993) J. Biol. Chem. 268, 22514–22519.

[12] Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22369–22377.
[13] Groettrup, M., Ruppert, T., Kuehn, L., Seeger, M., Standera, S., Koszinowski, U. and Kloetzel, P.M. (1995) J. Biol. Chem. 270, 23808–23815.
[14] Yang, Y., Früh, K., Ahn, K. and Peterson, P.A. (1995) J. Biol. Chem. 270, 27687–27694.
[15] Mott, J.D., Pramanik, B.C., Moomaw, C.R., Afendis, S.J., DeMartino, G.N. and Slaughter, C.A. (1994) J. Biol. Chem. 269, 31466–31471.
[16] Ahn, J.Y., Tanahashi, N., Akiyama, K.Y., Hisamatsu, H., Noda, C., Tanaka, K., Chung, C.H., Shimbara, N., Willy, P.J., Mott, J.D., Slaughter, C.A. and DeMartino, G.N. (1995) FEBS Lett. 366, 37–42.
[17] Kuehn, L. and Dahlmann, B. (1996) Arch. Biochem. Biophys. 329, 87–96.
[18] Kuehn, L. and Dahlmann, B. (1996) FEBS Lett. 394, 183–186.
[19] Gray, C.W., Slaughter, C.A. and DeMartino, G.N. (1994) J. Mol. Biol. 23, 67–75.
[20] Kania, M.A., DeMartino, G.N., Baumeister, W. and Goldberg, A.L. (1995) Eur. J. Biochem. 236, 510–516.
[21] Ahn, K., Erlander, M., Leturcq, D., Peterson, P.A., Früh, K. and Yang, Y. (1996) J. Biol. Chem. 271, 18237–18242.
[22] X. Song, J.D. Mott, J. von Kampen, B. Pramanik, K. Tanaka, C.A. Slaughter and G.N. DeMartino, J. Biol. Chem., 111 (1996) 26410-26417.
[23] Honore, B., Leffers, H., Madsen, P. and Celis, J.E. (1993) Eur. J. Biochem. 218, 421–430.
[24] Groettrup, M., Soza, A., Eggers, M., Kuehn, L., Dick, T.P., Schild, H., Rammensee, H.G., Koszinowski, U.H. and Kloetzel, P.M. (1996) Nature 381, 166–168.
[25] Bradford, M.M. (1974) Anal. Biochem. 72, 248–254.
[26] Dignam, J.D., Lebovitz, R. and Roeder, R.G. (1983) Nucl. Acids Res. 11, 1475–1489.
[27] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[28] Groettrup, M., Baron, A., Griffiths, G., Palacios, R. and von Boehmer, H. (1992) EMBO J. 11, 2735–2746.
[29] Negoescu, A., Labat-Moleur, F., Lorimier, P., Lamarcq, L., Guillermet, C., Chambaz, E. and Brambilla, E. (1994) J. Histochem. Cytochem. 42, (3) 433–437.
[30] Realini, C., Rogers, S.W. and Rechsteiner, M. (1994) FEBS Lett. 348, 109–113.
[31] Nikaido, T., Shimada, K., Shibata, M., Hata, M., Sakamoto, M., Takasaki, Y., Sato, C., Takahashi, T. and Nishida, Y. (1990) Clin. Exp. Immunol. 79, 209–214.
[32] Tojo, T., Kaburaki, J., Hayakawa, M., Okamoto, T., Tomii, M. and Homma, M. (1981) Ryumachi 2, 129–135.
[33] Realini, C., Dubiel, W., Pratt, G., Ferrell, K. and Rechsteiner, M. (1994) J. Biol. Chem. 269, 20727–20732.
[34] Tanaka, K. (1995) Mol. Biol. Rep. 21, 21–26.
[35] Frentzel, S., Kuhn-Hartmann, I., Gernold, M., Gött, P., Seelig, A. and Kloetzel, P.M. (1993) Eur. J. Biochem. 216, 119–126.
[36] Haass, C. and Kloetzel, P.-M. (1989) Exp. Cell Res. 180, 243–252.
[37] Li, N.X., Lerea, K.M. and Etlinger, J. (1996) Biochem. Biophys. Res. Commun. 225, 225–229.
[38] Yuan, X.Q., Miller, M. and Belote, J. (1996) Genetics 144, 147–157.
[39] Chu-Ping, M., Willy, P.J., Slaughter, C.A. and DeMartino, G.N. (1993) J. Biol. Chem. 268, 22514–22519.
[40] Haass, C., Pesold- Hurt, B., Multhaup, G., Beyreuther, K. and Kloetzel, P.M. (1989) EMBO J. 8, 2373–2379.
[41] Klein, U., Gernold, M. and Kloetzel, P.-M. (1990) J. Cell. Biol. 111, 2275–2282.
[42] Peters, J.M., Cejka, Z., Harris, J.R., Kleinschmidt, J.A. and Baumeister, W. (1993) J. Mol. Biol. 234, 932–937.